Electron Spin Resonance Studies of Wheat Protein Fractions

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Gluten was fractionated according to its extractability in dilute acid, generating fractions that differed in their gliadin/glutenin ratio and polymer composition. The fractions were characterized by SE-HPLC and by densitometry of electrophoregrams. The hydrated fractions were studied by electron spin resonance spectroscopy. Spin-probing was carried out with nitroxide radicals of various sizes, giving knowledge on the mobility and distribution of molecules in the system. The existence of at least two different aqueous environments was established within the system. Specific spin-labeling of the sulfhydryl or amine groups of protein enabled comparative studies of the rigidity of the protein chains. A factor of variation was found to be the quantity and size of prolamine, but this was not the only criterion.

Keywords: *Electron spin resonance; gluten; wheat storage proteins; protein aggregation*

INTRODUCTION

Bread-making quality of flours is related to the quantity and nature of glutenin subunits, essentially of high molecular weight (HMW) but also of low molecular weight (LMW) (Sadouki and Autran, 1987; He and Hoseney, 1992; Gupta et al., 1989; Payne et al., 1987a,b). Several approaches have been made to relate rheological properties and different quality criteria with glutenin subunit composition. The main methods used that do not alter the functional properties of the protein systems are as follows: (1) testing of random wheat lines, enabling one to make a nonexhaustive inventory of subunits and to correlate them to good and bad bread-making qualities (Branlard and Dardevet, 1985a,b; Graybosch et al., 1990; Payne et al., 1981); (2) obtaining and comparing near isogenic lines (NILs) differing by their composition in one or several glutenin subunits (Payne et al., 1987b; Lawrence et al., 1988); (3) using fractionation techniques of gluten systems that conserve the functionality of proteins, such as developed by MacRitchie (1987) by sequentially extracting gluten with dilute acid of increasing strength (MacRitchie et al., 1991). Such fractions have been characterized by size exclusion high-performance liquid chromatography (SE-HPLC) and rheological techniques (Lundh and MacRitchie, 1989; Cornec et al., 1994). The extractability of the fractions was found to correlate well with the size distribution of protein polymers and the quantities of HMW glutenin subunits.

The present work is concerned with functional gluten and gluten fractions. These systems were previously studied by SE-HPLC and dynamic rheology (Cornec et al., 1994). These authors showed that hydrated gluten proteins form a viscoelastic transient network. Viscoelasticity of the gluten fractions was strongly related to their large glutenin polymer content. It was assumed that these polymers are responsible for the connectivity of the network composed of gliadins and glutenin polymers. We used electron spin resonance (ESR) spectroscopy to characterize the solvent viscosity and the mobility of labels bound to various amino acid residues. The ESR techniques are well-known in polymer science and in the study of biological membranes and transient systems, but to our knowledge they have scarcely been used to study gluten proteins. Moonen et al. (1985) spin-labeled some reduced-alkylated HMW glutenin subunits. Functional hydrated networks were examined by spin-probing (Pearce et al., 1987, 1988; Nishiyama et al., 1981; Nishiyama and Kuninori, 1985) and by us for spin-labeling (Hargreaves et al., 1994a,b). This application becomes all the more interesting as it brings new elements to the characterization of gluten proteins on a molecular basis. ESR characterizes movements in the 10^{-8}–10^{-12} s range, reflecting the molecular organization of the protein aggregates and is therefore complementary to techniques such as microscopy and rheology, which reflect the network organization.

EXPERIMENTAL PROCEDURES

Fraction Preparation. Flour was from a French wheat cultivar, Aubaine, of good bread-making quality. The flour was defatted by two chloroform extractions and gluten was hand extracted with distilled water, freeze-dried, and ground to powder as described previously (Hargreaves et al., 1994a). Fractionation was done according to the method described by MacRitchie (1987), modified to get 12 fractions (Cornec et al., 1994). A 5 g aliquot of gluten powder underwent successive extractions with HCl as follows: fractions 1–3 (F1–F3) extracted by 100 mL of 0.3 mM HCl, fractions 4–6 (F4–F6) by 50 mL of 0.625 mM HCl, fractions 7–10 (F7–F10) by 50 mL of 1.5 mM HCl, fraction 11 (F11) by 50 mL of 5 mM HCl. The remaining insoluble material gave fraction 12 (F12). Extractions were carried out by homogenizing each mixture for 2 min at 5000 rpm with a Polytron PT 10/35 mixer; separation was done by centrifugation at 6000*g* for 15 min. The pH of the supernatant was adjusted to 5.8 with NaOH. The solution was then freeze-dried and ground to powder. This fractionation was carried out on five aliquots, and the resulting fraction series were pooled together. Fractions 1–3 were discarded as they did not give a dough-type structure upon hydration, but rather a solution or a gel, so ESR measurements could not be carried out on these fractions in similar conditions as for gluten. Fraction 12 was also discarded as it did not form a network, and its protein content was very low (42%).

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MacRitchie (1987) reconstituted flours with the different elements separated and found that mixograph peak development time and loaf volume were not significantly different from the control flour. This fractionation method was therefore presumed to preserve the functionality of the proteins.

Biochemical and Aggregative Characterization of Fractions. Protein contents were determined by the Kjeldahl method with a conversion factor of 5.7.

Proteins were characterized by sodium dodecyl sulfate 10–20% gradient polyacrylamide gel electrophoreses (SDS–PAGE) obtained in reducing conditions (Cornec et al., 1994), and relative proportions of protein bands were measured by densitometry.

Size exclusion high-performance liquid chromatography (SE-HPLC) was performed on a Kontron system 400 equipped with a Pharmacia HR column (1 × 30) with a Superose 6 Prep Grad gel (claimed separation domain: 5×10^3 to 5×10^6 Da). The eluting solution was borate buffer, 0.0125 M, pH 8.5, 0.1% SDS. Beforehand the samples were solubilized in buffer with 2% SDS by sonication (Cornec et al., 1994). Chromatograms were divided into four zones depending on their elution time: P1 corresponds to excluded large size glutenin polymers (>500 kDa), P2 to average size glutenin polymers (<500 and >70 kDa), P3 to gliadins (<70 and >15 kDa) and P4 to low molecular weight nonstorage proteins (<15 kDa).

The fatty acid content was determined by controlled hydrolysis and methylation of the samples, followed by gas chromatography measurements (Hargreaves et al., 1994a).

ESR Techniques. Hydrated gluten has no paramagnetic activity, so we employed spin probing and spin-labeling techniques, in which a compound with a nitroxide radical possessing a paramagnetic free electron is added to the system. We are therefore observing just one type of compound that can seek different environments in complex systems such as gluten.

In probing the paramagnetic compounds are not covalently linked. The ESR spectra reflect the motion of the small paramagnetic solute that depends on the probe size and the solvents viscosity. The size and the polarity of the probes influence their accessibility to microenvironments and their behavior in a network: a smaller probe may stay mobile were, for steric reasons, a larger molecule has a reduced mobility. The hydrodynamic radius (r) of spherical probes can be estimated by the Stokes–Einstein equation in solvents of known viscosity.

In spin-labeling the paramagnetic compounds are covalently linked to the polypeptide chain. The spectra will therefore reflect the motion of the labeled amino acid side chain, which is dependent on the local environment of the polypeptide chain. The influential parameters are the following: the spin label's reactant group which determines the amino acid residue the spin label will bind to; the radical's mobility, which increases with the length and flexibility of the spin-label molecule; and the size of the compound, which determines the accessibility to some amino acid residues and steric hindrance.

ESR Measurements. A Varian E9 spectrometer (Varian, Orsay, France) was used. Spectra were recorded with a 10 mW microwave power avoiding saturation phenomena. All nitroxide radicals were from Aldrich Chemicals (Strasbourg, France).

Spin-Probing and -Labeling of Protein Fractions. The nitroxide radicals used are represented in Figure 1. Except for TEMPO, the same compounds were used for probing and labeling experiments. In probing a large excess of nitroxide compound is used, so covalent interactions are negligible. In labeling, after an hour of reaction, the excess nitroxide is eliminated by extensive dialysis against distilled water, so only the covalently labeled nitroxide compounds will remain in the sample (Hargreaves et al., 1994a). The maleimido derivative was used to label the sulfhydryl groups and the isothiocyanato derivative was used to label the amino groups and possibly in a lesser way the hydroxyl groups. All ESR experiments were carried out in triplicate, and the mean value and standard deviation were calculated.

ESR Recordings and Calculations. The recording and calculation procedures used have been described elsewhere (Hargreaves et al., 1994a). The correlation time (τ_c) was

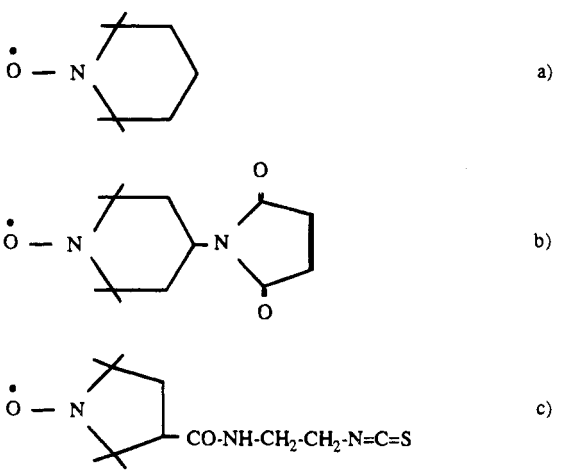

Figure 1. Structures of the paramagnetic compounds: (a) TEMPO; (b) 4-maleimido-TEMPO; (c) 3-[(2-isothiocyanatoethyl)carbamoyl]-PROXYL.

calculated from the features of the fast moving radicals ($\tau_c < 10^{-8}$ s) according to the theory of Freed and Frankel (1963). τ_c represents the time during which the nitroxide radical stays in a given orientation. When the spectra were composite—resulting from spin radicals in distinct environments—an estimation of the importance of the less mobile population was calculated by the ratio $R = i/m$, i and m being the amplitude of the low field peaks corresponding to the low and high mobility radicals, respectively.

RESULTS AND DISCUSSION

Biochemical and Aggregative Characteristics of Fractions. The protein contents of the freeze-dried fractions was about 80%. The composition of the fractions was analyzed by densitometry of SDS–PAGE electrophoregrams in reducing conditions (Table 1). In addition to storage proteins, the gluten comprised some nonstorage proteins (NSP), which were approximated for convenience to the low molecular weight nonstorage proteins (14 000–17 000). Fractions 5–7 had higher NSP contents. The fractions differing in their extractabilities exhibited different gliadin/glutenin composition, with an increase in the content in HMW glutenin subunits from F4 to F11. The densitometry data were confirmed by the SE-HPLC results which show that the content in large polymers of glutenin (P1) increases from 16.5 to 52.7% while the gliadin content (P3) decreases from 40.9 to 9.3% between F4 and F11 (Table 2). The relative content in small glutenin polymers (P2) is highest in the intermediate fractions, while the relative content of low molecular weight NSP (P4) does not follow any pattern, but is higher in F5–F8.

The contents in NSP determined by densitometry and by SE-HPLC are approximate values, as their amino acid composition differs from that of storage proteins. They will fix more Blue Coomassie dye and will have a different absorbance (Fullington et al., 1980). However, our data enable comparisons of NSP contents between fractions.

The lipid content distribution did not vary much or seem to follow any specific pattern, although the lipid content was slightly increased in the intermediate fractions (Table 1).

Spin-Probing with TEMPO. The TEMPO spin-probing of the samples gave a three-line spectra representative of nitroxide radicals in a polar liquid medium. There was no high field doublet characteristic of probes in two media differing by there polarities, such as membranes. The chloroform washing of flour totally extracted the lipid phase responsible for the observation

Table 1. Composition of the Gluten Fractions in Solubility in HCl[a]

	HMW glutenin subunits (SU)[b]	HMW albumin and ω-gliadins[b]	gliadins and LMW glutenin SU[b]	NSP[b]	fatty acid (mg/100 mg)
MW	94 000	67 000	30 000 to 43 000	14 000 to 16 500	
F4	13.5	12.2	59.3	12.9	0.54
F5	13.0	10.8	43.6	30.6	0.86
F6	18.8	14.3	39.5	24.4	1.21
F7	17.7	14.5	41.3	22.4	1.01
F8	20.8	14.7	43.5	16.1	0.84
F9	22.2	13.8	43.2	16.3	1.16
F10	22.1	12.2	48.8	11.6	1.15
F11	24.3	12.1	47.0	9.7	0.61

[a] Protein characterization by densitometry of SDS–PAGE electrophoregrams was obtained in reducing conditions; fatty acid content was determined by GC of hydrolyzed, methylated samples; internal standard is C17:0. [b] Relative area determined by densitometry.

Table 2. Aggregative State of Gluten Fractions Observed by SE-HPLC, after Solubilization by Sonication[a]

	P1	P2	P3	P4
F4	16.44	29.62	40.93	12.98
F5	12.07	34.66	32.70	20.56
F6	19.26	36.09	24.19	20.34
F7	20.20	42.26	16.92	20.60
F8	20.38	41.12	17.33	21.16
F9	24.90	41.06	15.75	18.30
F10	46.58	29.27	11.83	12.36
F11	52.74	23.48	9.27	14.63

[a] Data are presented as relative areas: P1, large size glutenin polymers; P2, average size glutenin polymers; P3, gliadin; P4, low molecular weight nonstorage proteins.

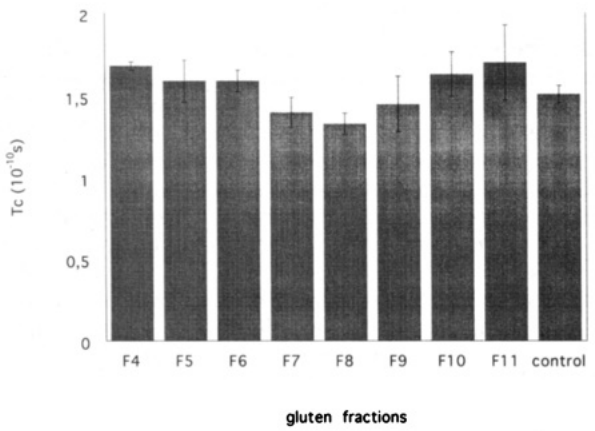

Figure 2. Correlation times (τ_c) of gluten fractions obtained by spin-probing with TEMPO.

of this phenomenon in gluten. The remaining lipids (Table 1) are therefore probably not in an organized phase but dispersed in the system (Pearce et al., 1988; Hargreaves et al., 1994a). The correlation time of TEMPO in the control gluten (1.5×10^{-10} s) was significantly higher than in water (0.2×10^{-10} s). These values enable the estimation of the average solvent viscosity of the system by the Stokes–Einstein equation. The value thus obtained in the hydrated gluten system was 8.2×10^{-3} Pa·s. The presence in the aqueous phase of some macromolecules, protein and carbohydrate, may be responsible for variation. There is no slow motion probe, so we can presume there is no or negligible steric hindrance of TEMPO (diameter about 0.52 nm) in the system. There was no significant difference between the correlation times of the fractions, although the general aspect of Figure 2 seems to show the probe in F8 as having the lowest mobility.

Spin-Probing with the Maleimido Derivative. The spectra obtained with the different gluten fractions show the existence of at least two populations of nitroxide radicals differing by their mobility (Figures 3a and 4). A fraction of the probe was therefore "immobilized" by the system, signifying there is some steric hindrance, possibly intermolecular spaces in the protein aggregates of similar dimension as the probes' hydrodynamic diameter (about 0.76 nm). Values of R and τ_c tended to diminish when the glutenin/gliadin ratio increased, fraction 4 having the highest values. This could be attributed to the lower content of glutenin polymers in this fraction, the gliadins ensuring a higher continuity of the system, with a smaller volume of water cavities.

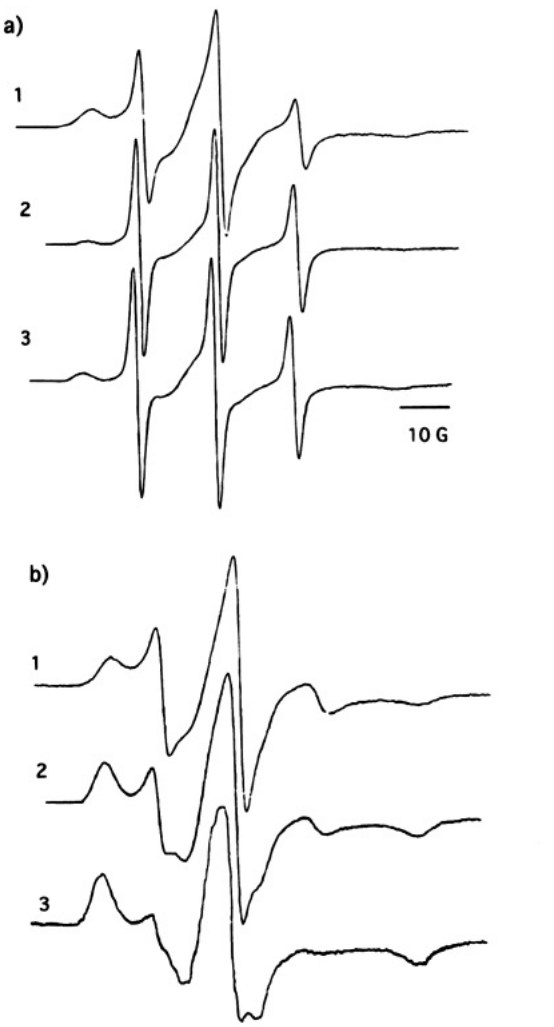

Figure 3. Examples of ESR spectra obtained with 4-maleimido-TEMPO (a) by spin-probing and (b) by spin-labeling. 1, fraction 4 (rich in gliadin); 2, fraction 8 (intermediate), 3, fraction 11 (rich in large glutenin polymers).

Spin-Probing with the Isothiocyanato Derivative. The probe used in this experiment is larger than the previous ones (Figure 1). The probing results show

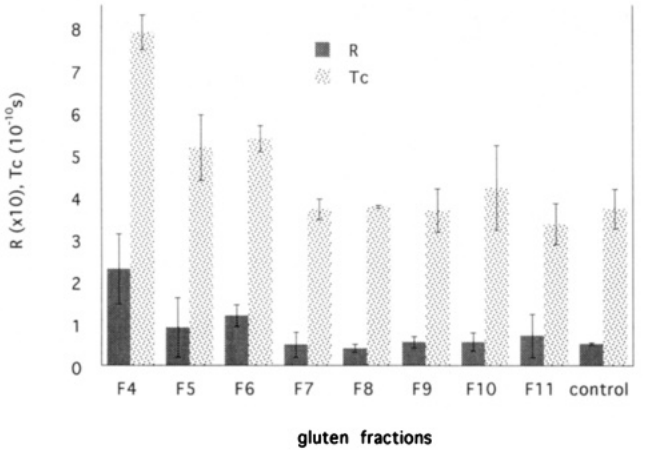

Figure 4. R values and correlation times (τ_c) of gluten fractions obtained by spin-probing with 4-maleimido-TEMPO.

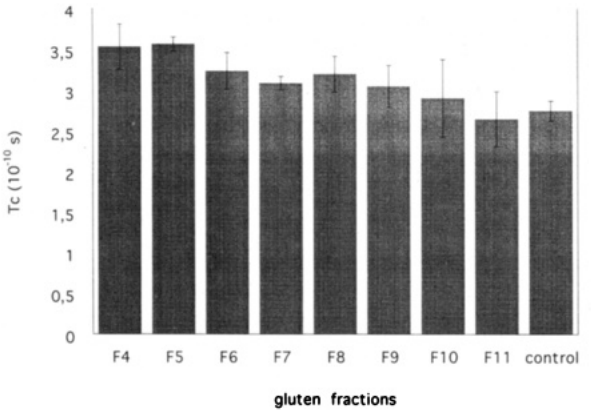

Figure 5. Correlation times (τ_c) of gluten fractions obtained by spin-probing with the isothiocyanate derivative.

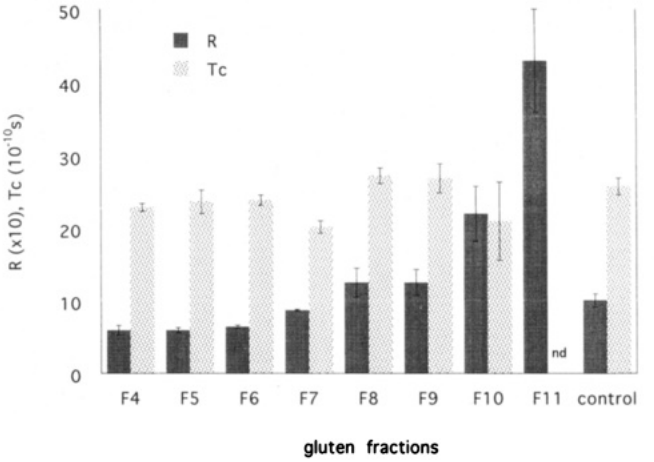

Figure 6. R values and correlation times (τ_c) of gluten fractions obtained by spin-labeling with 4-maleimido-TEMPO.

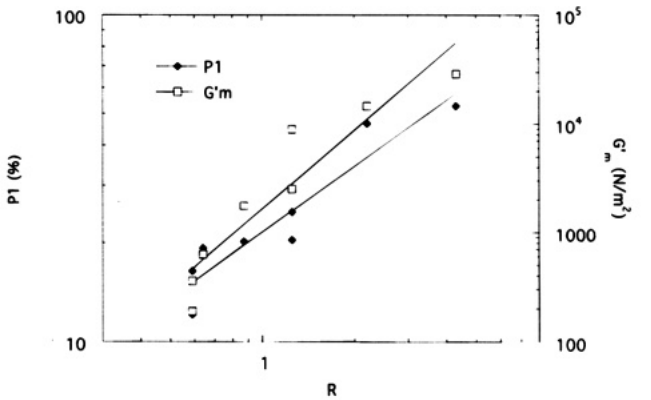

Figure 7. Relation between the content of gluten fractions in very large glutenin polymers (P1), their plateau modulus (G'_m), and their R value obtained by spin-labeling of the cysteine residues.

only traces of slow motion population ($R \approx 0$), which can be interpreted as meaning that the probe is excluded from the glutenin and gliadin aggregates. Thus the probe is thought to be in the water phase or pockets of the system, although the τ_c values are higher than in a water solution (0.6×10^{-10}s). τ_c did not vary significantly between samples (Figure 5), but the general aspect suggests a slight increase in mobility when increasing the glutenin/gliadin ratio, as was the case with the previous probe.

Spin-Labeling of the Sulfhydryl Groups. As the systems studied are complex, involving a large number of nonstorage proteins (NSP), it was previously ascertained that the gluten systems studied reflected the behavior of the storage proteins by comparing them to NSP extracted glutens (Hargreaves et al., 1994b). The NSP did not seem to account for large variations in spin-labeling experiments with 4-maleimido-TEMPO.

The R value increased with the glutenin/gliadin ratio (Figures 3b and 6), but this variation cannot be explained only from the evolution of this ratio (Table 1). This increase in R value is related to the content in large glutenin polymers (P1) (Figure 7), which would indicate that the cysteine environment of these polymers is very rigid. The cross-linking of glutenin subunits to form polymers reduces the mobility of the cysteine environment of gluten proteins. Cornec et al. (1994) determined by frequency sweeps the elastic plateau modulus (G'_m) of the gluten fractions, which is proportional to the connectivity of the gluten transient network. It was increased when the content of gluten fractions in P1 increased. We found that the R values correlated strongly with G'_m (correlation coefficient of 0.94) (Figure 7). This shows the proportion of "immobile" spin labels situated on cysteine residues evolves with the connectivity of the systems studied although the range of movements observed by ESR spectroscopy and dynamic rheology are very different, due to the observation times (nanoseconds and 10ths of seconds, respectively). Less mobile side chains correspond to an increased rigidity of the protein network. This "immobilization" could be due to the involvement in weak interactions of the intimate environment of the labeled cysteine residues.

The τ_c values reflecting the motion of the more mobile labels are not significantly different and could be attributed to the polypeptides that are not involved in the glutenin polymers P1.

Spin-Labeling of the Amino Groups. Spin-labeling with 3-[(2-isothiocyanatoethyl)carbamoyl]-PROXYL (Figure 1) resulted in complex spectra. The R value differed between fractions, with a general aspect pointing to a maximum in F8 (Figure 8). Unlike the sulfhydryl spin-labeling, no relation can be found with a given gluten component or SE-HPLC fraction; it is therefore suggested that the variations in R values are due to differences in the organization of the gluten fractions. The amino groups labeled are in a particularly rigid environment, spin-labeling experiments with shorter isothiocyanate derivatives showing powder type spectra (Hargreaves et al., 1994b). Amino groups are found on lysine residues and on N-terminal amino acids. Lysine residues have been reported to be in the alpha-helix rich domains where disulfide bonds are located (Shewry et al., 1992). The τ_c values of the mobile fraction were not significantly different between samples.

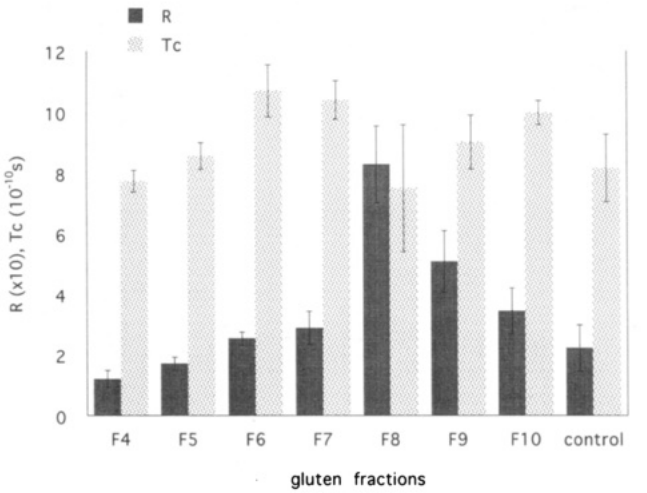

Figure 8. R values and correlation times (τ_c) of gluten fractions obtained by spin-labeling with the isothiocyanate derivative.

CONCLUSION

This study shows that spin-probing may reflect different parts of the gluten systems, the smaller probes having access to the intra-aggregate aqueous phase while the larger ones are restricted to the inter-aggregate water phase which includes "water pockets". The continuity of this inter-aggregate space seems to be related to the gliadin content. Spin-labeling of the cysteine residues showed an increase in rigidity of protein segments correlated to the amount of largest glutenin polymers (P1) determined by SE-HPLC and which can also be related to the connectivity and the viscoelasticity of the protein network. Results from spin-labeling of the amino groups seem to reflect a difference of organization of the gluten network. These observations at a molecular level can be linked to results showing that intermediate type fractions are generally the best improvers of loaf volume upon addition of 1% to flour (MacRitchie, 1987), and this for fractions from different bread-quality wheats. Spin-labeling could therefore enable selective observation of molecular variations in gluten proteins from a compositional and structural point of view and from an organizational aspect. Further research is being carried out to better understand the link between molecular properties observed by ESR and functionality of hydrated gluten.

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